Severe pyridine nucleotide depletion in fibroblasts from Lesch–Nyhan patients

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The relationship between a complete deficiency of the purine enzyme hypoxanthine-guanine phosphoribosyltransferase and the neurobehavioural abnormalities in Lesch–Nyhan disease remains an enigma. *In vitro* studies using lymphoblasts or fibroblasts have evaluated purine and pyrimidine metabolism with conflicting results. This study focused on pyridine nucleotide metabolism in control and Lesch–Nyhan fibroblasts using radiolabelled salvage precursors to couple the extent of uptake with endocellular nucleotide concentrations. The novel finding, highlighted by specific culture conditions, was a marked NAD depletion in Lesch–Nyhan fibroblasts. ATP and GTP were also 50 % of the control, as reported in lymphoblasts. A 6-fold greater incorporation of [14C]nicotinic acid into nicotinic acid–adenine dinucleotide by Lesch–Nyhan fibroblasts, with no unmetabolized substrate (20 % in controls), supported disturbed pyridine metabolism, NAD depletion being related to utilization by poly(ADP-ribose) polymerase in DNA repair. Although pyrimidine nucleotide concentrations were similar to controls, Lesch–Nyhan cells showed reduced [14C]cytidine/uridine salvage into UDP sugars. Incorporation of [14C]uridine into CTP by both was minimal, with more than 50 % [14C]cytidine metabolized to UTP, indicating that fibroblasts, unlike lymphoblasts, lack active CTP synthetase, but possess cytidine deaminase. Restricted culture conditions may be neccesary to mimic the situation in human brain cells at an early developmental stage. Cell type may be equally important. NAD plus ATP depletion in developing brain could restrict DNA repair, leading to neuronal damage/loss by apoptosis, and, with GTP depletion, affect neurotransmitter synthesis and basal ganglia dopaminergic neuronal systems. Thus aberrant pyridine nucleotide metabolism could play a vital role in the pathophysiology of Lesch–Nyhan disease.

Key words: Lesch–Nyhan disease, NAD, neurological deficit, purine, pyrimidine nucleotide.

INTRODUCTION

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is an essential 'salvage' enzyme which recycles the purine bases guanine and hypoxanthine to nucleotides in human cells, thereby sparing *de novo* synthesis [1]. Although highest activity is found in human brain, efforts to equate the virtual absence of HPRT in cells and tissues of children with the devastating Lesch–Nyhan disease (LND) with the neurological deficits characterizing this X-linked disorder have met with scant success [1–3]. Classic LND, as well as LND variants, are also characterized by gross uric acid overproduction and frequently present first with renal failure and/or gout [1,4]. Urolithiasis as well as gout may be the main presenting symptom in patients with partial HPRT deficiency, Kelley–Seegmiller syndrome or KSS, who share the gross uric acid overproduction but lack neurological deficits [1,3,5]. The mild to absent neurological symptoms in such variants clearly implicates defective HPRT activity in the fulminant LND [1,3]. Until now, the underlying cellular and molecular mechanisms have remained largely unexplained and there is no effective treatment for the neurological deficits. Many LND boys do not survive beyond their 20s, dying from respiratory dysfunction [6]. Although HPRT-knockout (HPRT−) mouse models showed the characteristic gross purine overproduction, neurological abnormalities were absent originally [7]. Recent reports of a loss of basal ganglion dopamine, dependent on the age and strain and the subregion examined, accord with post-mortem studies in LND brain [8,9]. The absence of detectable morphological changes in the nervous system at autopsy in LND, even by electron microscopy, has been equally puzzling [7,10]. Here too, new studies using a variety of imaging techniques have highlighted hitherto undetectable abnormalities, including confirming the deficits in dopaminergic function reported [11,12].

To address the role of HPRT in the pathogenesis of LND, in lieu of brain tissue, investigators have employed *in vitro* studies to compare nucleotide synthesis in different cell types (lymphoblasts/fibroblasts) from LND patients versus subjects with normal HPRT activity, or tissue/cell lines from HPRT-knockout rodent models. The results (Table 1) have been controversial [6,13–22]. Striking increases in pyrimidine nucleotides were reported originally in HPRT− lymphoblasts, but not in fibroblasts, ATP and GTP being normal in both [16,17], whereas McCreanor and Harkness [6] noted lower purine nucleotides in LND lymphoblasts (Table 1). Other investigators have reported normal or even increased concentrations of purine, or purine plus pyrimidine, nucleotides in HPRT− fibroblasts [1,21]. Early studies in brain tissues from HPRT− mice showed normal ATP and GTP content (Table 1), consistent with the absence of overt neurological abnormalities [7]. Other workers using cultured astroglia from HPRT− mice reported reduced purine and elevated pyrimidine nucleotides, but normal purine and elevated pyrimidine nucleotides in an HPRT− rat neuroma line [19]. Normal purine but elevated pyrimidine nucleotides were reported subsequently in cultured astroglia and neurons from HPRT− transgenic mice [20,22]. Unfortunately, in some instances absolute values for nucleotides were not given [20,21].

Table 1   Mean purine and pyrimidine nucleotide concentrations reported in cells from LND patients and HPRT⁻ cells relative to controls

Mean ATP, GTP, UTP, CTP concentrations reported for HPRT⁻ cells (human fibroblasts and lymphoblasts, or murine and rodent cells) compared with controls vary considerably with the cell type used. −, not given.

Model	Concentration (mean ± S.D.)				
	ATP	GTP	UTP	CTP	Reference
Fibroblast ($n = 4$)					
LND*	2.72	0.572	−	−	[13]
Control*	2.13	0.577	−	−	
Fibroblast ($n = 3$)					
LND†	9.0	2.0	8.2‡	‡	[17]
Control†	11.8	2.5	8.0‡	‡	
Fibroblast ($n = 5$)					
Control†	13.7 ± 4.2	4.9 ± 1.7	4.2 ± 1.1	1.2 ± 0.6	[23]
Lymphoblast					
LND ($n = 11$)§	11.84 ± 2.23	3.16 ± 0.84	−	−	[18]
Control ($n = 7$)§	15.4 ± 1.17	4.5 ± 82	−	−	
Lymphoblast ($n = 6$)					
LND†	3.65	0.78	1.67	0.15	[16]
Control†	3.4	0.62	0.8	0.09	
WI-L2 ($n = 6$)					
HPRT⁻†	5.1 ± 0.42	1.36 ± 0.1	2.25 ± 0.16	0.64 ± 0.01	[16]
Control†	3.96 ± 0.26	1.17 ± 0.09	1.43 ± 0.1	0.19 ± 0.02	
Mouse brain ($n = 6$)					
HPRT⁻	10.9 ± 1.2	1.4 ± 0.1	−	−	[7]
Control	9.8 ± 1.0	1.5 ± 0.1	−	−	
Rat neuroma line ($n = 13$)					
HPRT⁻§	35.965 ± 9.36	4.439 ± 1.91	8.99 ± 2.6	−	[19]
Control§	41.945 ± 9.13	5.8 ± 1.93	6.97 ± 1.48	−	

* Units: μmol/g of protein.
† Units: nmol/10⁶ cells.
‡ Total pyrimidine nucleotides only.
§ Units: nmol/mg of protein.
¶ Units: pmol/mg of protein.

Contributing factors to explain such variations by careful investigators using the same cell types have included culture conditions, plus unappreciated differences in growth rates, glutamine deprivation, and the availability of 5-phosphoribosyl-1-pyrophosphate (PPRibP) [6,14–16,23]. Accelerated PPRibP generation and purine synthesis *de novo* can both be demonstrated in cultured cells at high phosphate concentrations [14,15]. Undialysed, non-heat-inactivated foetal bovine serum (FBS) contains purine- and pyrimidine-degrading enzymes plus high concentrations of nucleosides and bases, changing culture conditions [14,24–27]. The relevance of growth rate as well as culture conditions is underlined by their role in defining a developmental disorder associated with increased cellular nucleotidase activity [23]. Fast-growing fibroblasts have a much higher nucleotide content per cell than the slowest-growing cells. Passage number may also affect nucleotide concentrations, as can harvesting fibroblasts in the logarithmic phase of growth, or at confluence [23].

Our interest in possible derangements in pyridine nucleotide metabolism in LND stemmed from our earlier findings in the erythrocytes of LND patients of elevated NAD, as well as low GTP and elevated UDP sugars (UDPS) [25]. Our more recent studies in HPRT⁻ fibroblast lines in which HPRT activity had been restored by adenoviral transfection showed that such restoration increased GTP and reduced UDPS concentrations in the human HPRT⁻ fibroblast line, but not in the murine line [26]. We established that these differences related to xanthine dehydrogenase activity in the murine cells, but not the human cells, confirming that rodents are not an ideal model for defining pathologies involving human purine metabolism [26].

In addition, since most studies involving the use of radio-labelled substrates to compare synthesis and salvage in LND cells had focused on purine metabolism [1] we investigated pyrimidine, as well as pyridine, salvage pathways, using radio-labelled precursors to couple the extent of uptake with actual endocellular nucleotide concentrations. Because of the spurious effects of nucleosides, bases and enzymes abundant in undialysed non-heat-inactivated serum, of glutamine deprivation, or phosphate on PPRibP concentrations [14,15,23,24,27], plus variability in precursor concentrations (e.g. nicotinamide) in different culture media, appropriate precautions were incorporated in the experimental design. Under these carefully controlled conditions we found that LND fibroblast NAD concentrations were low compared with control fibroblasts, not elevated as found in LND erythrocytes [25].

EXPERIMENTAL

Chemicals

Trichloroacetic acid (TCA) and all chemicals or standards for HPLC (Analar or Aristar grade), [¹⁴C]uridine (0.9 mM, 56 mCi/mmol) and [¹⁴C]cytidine (0.91 mM, 55 mCi/mmol) were from Sigma, Poole, Dorset, U.K., and [¹⁴C]nicotinic acid (0.91 mM, 55 mCi/mmol) was from Amersham Bioscience. RPMI 1640, Hanks balanced salt solution, heat-inactivated FBS, RPMI 1600 and penicillin/streptomycin (10000 units/ml and 10000 μg/ml) were all from Gibco, Paisley, Scotland, U.K. The FBS was dialysed for 12 h against several changes of isotonic saline.

Table 2   Severe ATP, GTP and NAD depletion in fibroblasts from LND patients extracted directly or after 2 h incubation as described in the Experimental section

Mean NAD, ATP and GTP, but not UTP or CTP, concentrations ($\pm$ S.D.) are significantly lower in LND fibroblasts compared with controls either extracted directly, or cultured in full medium for a further 2 h, with or without the ^{14}C-labelled substrates listed in Figures 1, 3 and 4. **, Highly significant; n.s., not significant.

		Concentration (pmol/10^6 cells; mean $\pm$ S.D.)			
		LND		Control	
	Time …	0 h ($n = 8$)	2 h ($n = 5$)	0 h ($n = 8$)	2 h ($n = 5$)
ATP**		5883 ± 1414 $P < 0.0001$	4767 ± 1826 $P < 0.0001$	$12\,542 \pm 2265$	9788 ± 258
GTP**		1143 ± 325 $P < 0.003$	1146 ± 401 $P < 0.0039$	2363 ± 470	2165 ± 536
NAD**		942 ± 243 $P < 0.0001$	847 ± 312 $P < 0.0007$	1734 ± 272	1560 ± 177
UTP (n.s.)		2647 ± 674 $P = 0.08$	1937 ± 618 $P = 0.16$	3507 ± 1097	2636 ± 953
CTP (n.s.)		758 ± 322 $P = 0.54$	870 ± 501 $P = 0.4$	1089 ± 309	1083 ± 329

Subjects

The fibroblasts used (passage numbers 7–11) were grown from lines established by skin biopsy from seven LND patients who fulfilled the biochemical criteria of no demonstrable HPRT activity in intact or lysed cells. Cell lines established from subjects with no neurological disease were used for comparison. One LND line and one control line were grown up each week over an 8 week period in Ham's F10 medium, containing 2 % L-glutamine, 2 % antibiotics (penicillin + streptomycin) and 15 % FBS. The cell lines were seeded into 75 cm² tissue culture flasks and grown to 50 % confluence. The majority of these fibroblasts were used to study the activity of enzymes of pyridine nucleotide metabolism (G. Jacomelli, S. Sestini, L. D. Fairbanks, V. Micheli, L. Notarantonio, B. Cerboni, H. A. Simmonds and G. Pompucci, unpublished work). The remainder were subcultured as follows.

Cell culture

The above fibroblasts from the seven LND patients and controls were subcultured in RPMI 1640 + 10 % FBS, with medium being changed every 3–4 days and the cultures supplemented daily with 1 mM glutamine. Duplicate cultures were also grown up from one LND and one control, bringing the total number of experiments to eight. Growth rate was slow, presumably because of the small number of cells seeded initially. At subconfluency both HPRT⁻ and control cells were washed twice with PBS (without Mg^{2+} and Ca^{2+}) and detached from the flasks by trypsinization, the trypsin being inactivated by adding RPMI + FBS. Fibroblasts were washed in the above medium, counted and resuspended at approx. $(1–2) \times 10^6$ cells/ml and used immediately for the studies described below.

Nucleotide extracts

An extract was made from both the above HPRT⁻ and control cells. In addition, in five of the eight experiments, fibroblasts were incubated for 2 h in their own medium without any radiolabel for comparison with the nucleotide concentrations in the 2 h pulse-labelling studies. In either case cells were centrifuged in a microfuge at 1000 **g** for 1 min and the cell pellet disrupted immediately with 200 μl of 10 % TCA. Precipitated protein was removed by centrifugation at 12000 **g** and the TCA back-extracted from the supernatant with water-saturated diethylether to a pH of 5.0. Extracts, if not processed immediately, were frozen at −20 °C for subsequent analysis and the pellets retained for protein estimation by the Lowry method.

Pulse-labelling studies

Fibroblasts [$(0.2–0.4) \times 10^6$ cells] were incubated in 1.5 ml sterile Eppendorf tubes containing 200 μl of RPMI plus 10 % heat-inactivated dialysed FBS and antibiotics, as described above, with the appropriate radiolabelled substrate as follows [26,27]. (i) [^{14}C]Nicotinic acid (25 μM) was used to evaluate pyridine nucleotide pathways in five experiments. (ii) [^{14}C]Uridine (25 μM) and [^{14}C]cytidine (25 μM) were used in these five experiments, and in three additional experiments, to evaluate pyrimidine nucleotide salvage pathways.

Reactions were commenced by the addition of the particular ^{14}C-labelled substrate, the incubations being carried out in a shaking water bath for 2 h at 37 °C. After 2 h cells were centrifuged and TCA extracts prepared as described above.

HPLC analyses

A Waters Trimodular system that incorporated in-line photo-diode array and radiodetection was used for the separation and quantification of nucleotides [26,27]. Cell extract (100 μl) was injected on to a Phenomenex Hypersil 5 μm NH2-2 column (250 mm × 3.2 mm) at a flow rate of 0.5 ml/min using a linear phosphate-buffer-gradient elution system. Buffer A was 5 mM KH_2PO_4, pH 2.65, and buffer B was 0.5 M KH_2PO_4/1.0 M KCl, pH 3.8. The limit of detection was 0.1 pmol/10^6 cells. Quantification of ribonucleoside mono-, di- and tri-phosphates was made from their characteristic UV absorption spectra and retention time compared with a mixture of 17 authentic standards. The rate and route of incorporation of ^{14}C radiolabel into the different nucleotide pools was followed using an in-line radiodetector (Reeve Analytical, Glasgow, U.K.). Data collection and processing was performed using Waters Millennium software; results were calculated with a Lotus 1-2-3 spreadsheet. Statistics were evaluated by ANOVA using the Instat package.
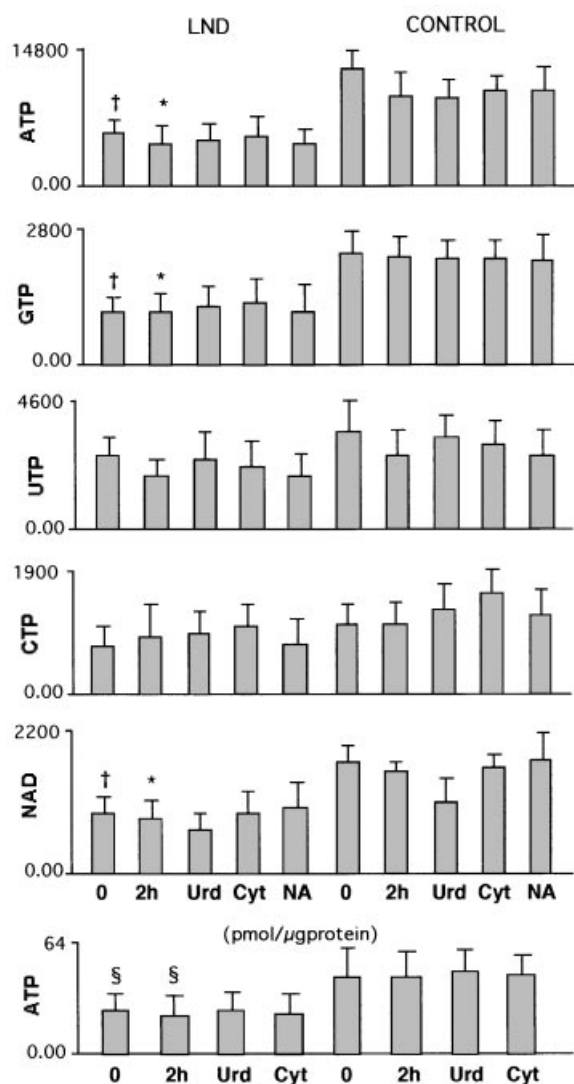
Figure 1 Mean fibroblast nucleotide concentrations

There was a high significance of differences between NAD, ATP and GTP concentrations (pmol/10^6 cells; mean $\pm$ S.D.) in HPRT$^-$ fibroblasts (left), compared with controls (right), but not between UTP or CTP extracted directly (0 h), or cultured for 2 h with or without the radiolabelled substrates indicated (Urd, uridine; Cyt, cytidine; NA, nicotinic acid). Note the significant reduction in NAD in either HPRT$^-$ or control fibroblasts when incubated with uridine. Depletion of NAD, ATP and GTP in HPRT$^-$ cells (bottom panel, shown for ATP only) was still highly significant when compared on a protein basis (pmol/μg of protein): $\dagger P < 0.0001$; $^*P < 0.005$; $\S P < 0.01$, compared with the relevant control.

RESULTS

Nucleotide concentrations in control and LND fibroblasts

Mean purine, pyrimidine and pyridine triphosphate nucleotide pools in fibroblasts extracted before ($n = 8$) or after incubation for 2 h alone ($n = 5$) are shown in Table 2.

Control fibroblast nucleotide concentrations

Mean ATP, UTP and CTP concentrations in control extracts made directly, i.e. prior to incubation (Table 2), were similar to those reported by Page et al. [23], the energy charge for the adenine nucleotides (ATP:ADP:AMP) being high (approx.
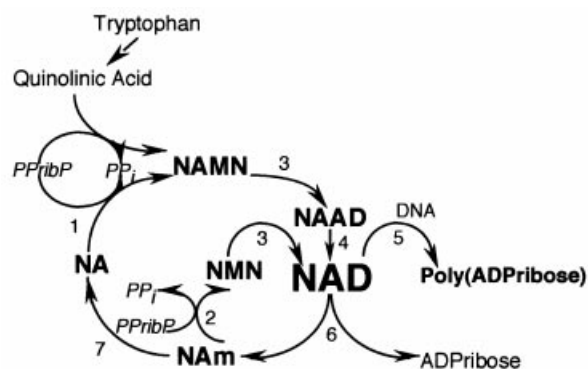
Figure 2 Routes of NAD formation, degradation and utilization in humans

The pyridine nucleotide cycle highlighting the importance of PPRibP in the routes of NAD synthesis from either the tryptophan pathway, or nicotinic acid (NA) and nicotinamide (NAm), and the enzymes involved in its utilization either for poly(ADP-ribose) synthesis, or breakdown to nicotinamide and ADP ribose. NAMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide. Enzymes: 1, nicotinic acid phosphoribosyltransferase (E.C. 2.4.2.11); 2, nicotinamide phosphoribosyltransferase (E.C. 2.4.2.12); 3, nicotinic acid mononucleotide adenylyltransferase (E.C. 2.7.7.18) and nicotinamide mononucleotide adenylyltransferase (E.C. 2.7.7.1); 4, NAD synthetase (E.C. 6.3.5.1); 5, poly(ADP-ribose) synthetase (E.C. 2.4.2.30); 6, NAD glycohydrolase (E.C. 3.2.2.6); 7, nicotinamide deamidase (E.C. 3.5.1.19).

10:1.0:0.1; results not shown). However, control GTP concentrations in our study were about 50 % of those reported (Table 1). No NAD concentrations have been listed for fibroblasts (Table 1), values being given for lymphoblasts in a single study only (LND, 2.18 ± 0.35 nmol/mg of protein; control, 2.92 ± 0.98 nmol/mg of protein) [7].

Slight differences between mean nucleotide pools in non-incubated cells compared with cells incubated for 2 h were not significant. Nucleotide concentrations were generally slightly lower, presumably reflecting loss of damaged cells, with re-utilization of nucleosides or bases for essential intermediates in glycosylation or lipid synthesis [27].

LND fibroblast nucleotide concentrations

Differences in mean nucleotide pools found between non-incubated and incubated LND cells, likewise, were not significant (Table 2) and the energy charge for adenine nucleotides (ATP:ADP:AMP) was also high, approx. 10:1.0:0.1 (results not shown). However, mean NAD, ATP and GTP concentrations in LND cells were consistently around 50 % of control means. These differences were striking, as confirmed by the statistical significance according to ANOVA. The two-tailed P values for the means for NAD ($P < 0.0001$ direct, $P < 0.001$ incubated) were extremely significant (Table 2). Likewise, differences in mean ATP and GTP concentrations compared with controls (Table 2) were extremely significant for both the non-incubated and incubated extracts ($P < 0.0001$), and very significant for GTP in both non-incubated and incubated extracts ($P < 0.003$ and $P < 0.004$, respectively). However, corresponding P values for either non-incubated or incubated LND compared with control cells for UTP ($P = 0.08$ and 0.16, respectively) and CTP ($P = 0.54$ and 0.4, respectively) were not significant (Figure 1 and Table 2). Thus, both pyridine and purine nucleotides concentrations in fibroblasts from LND patients were very low compared with controls, but pyrimidines were not significantly different.
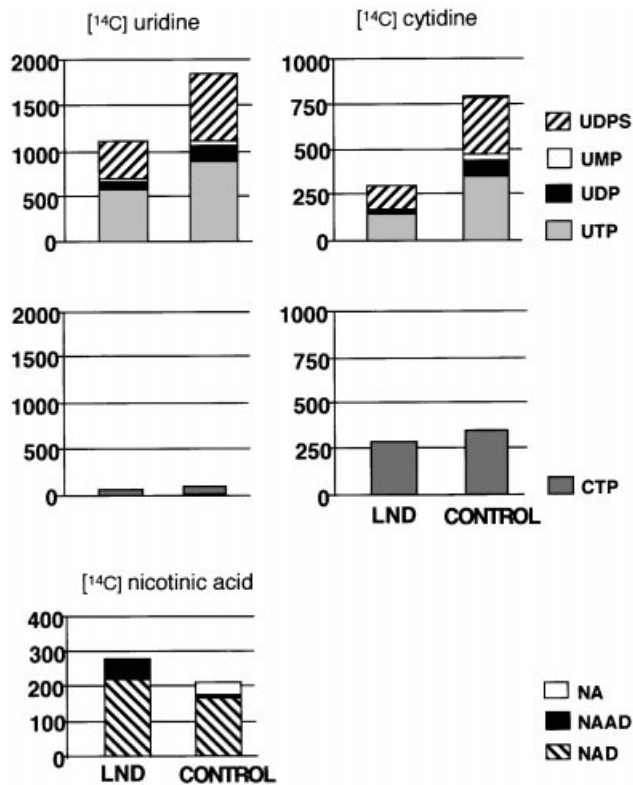
Figure 3   Mean ^{14}C incorporation into fibroblasts

Accelerated mean incorporation (pmol/10^6 cells per 2 h) of [^{14}C]nicotinic acid (NA) into NAAD and NAD by HPRT$^-$ fibroblasts compared with controls (bottom panel; $n = 5$), the reverse being the case for [^{14}C]uridine (left-hand top and middle panels) and [^{14}C]cytidine (right-hand top and middle panels). Notably, very little [^{14}C]uridine is incorporated into CTP by either HPRT$^-$ or control cells. By contrast, approx. 50% of [^{14}C]cytidine is incorporated into CTP by HPRT$^-$ cells, the remainder being deaminated and incorporated into uridine nucleotides (UTP, UDP, UMP or UDPS), whereas less is salvaged into CTP and more deaminated by controls ($n = 8$).

Fibroblast nucleotide triphosphate concentrations

To ensure that the above results were not an artefact of counting variations, nucleotide triphosphates were also calculated on a protein basis. The bottom panel in Figure 1 confirms that mean ATP concentrations (pmol/μg of protein) in LND fibroblasts were likewise significantly different from controls ($P < 0.05$). Although the results are shown for ATP only, a similar significance was found for GTP and NAD, whereas the values for UTP and CTP were not significant ($P = 0.9$ and 0.8 respectively).

Nucleotide concentrations in pulse-labelling studies

Slight increments in mean nucleotide concentrations occurred with the different ^{14}C-radiolabelled substrates (25 μM), compared with cells incubated alone (Figure 1), but the changes were not significant. NAD, ATP and GTP concentrations were still significantly lower in LND cells compared with controls for each substrate (range, $P \leqslant 0.01$–$P < 0.0005$), with no significant difference between pyrimidine pools.

A notable result was that uridine actually reduced NAD concentration, not only in LND cells (by 17%, although this failed to reach significance), but also in controls where NAD fell by 29% ($P < 0.01$). There was no increase in ADP ribose in either control or LND cells, which excludes NAD breakdown by
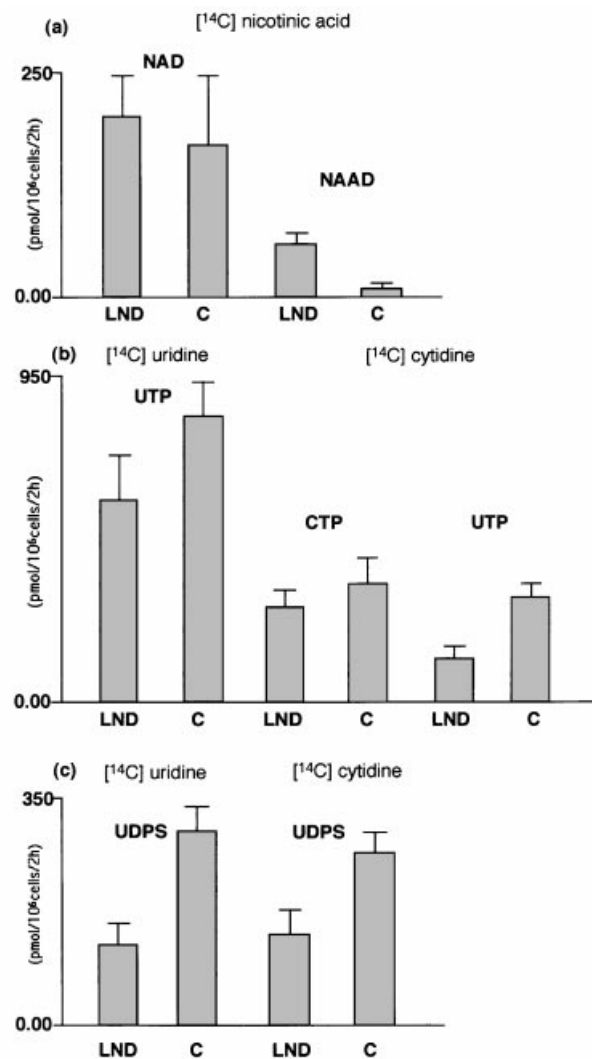

Figure 4   Mean ^{14}C incorporation into LND fibroblasts and controls

The same data as in Figure 3 reported as means $\pm$ S.D. comparing incorporation of radiolabelled substrates by LND fibroblasts (LND) and controls (C), showing that the difference between the incorporation of: (**a**) [^{14}C]nicotinic acid into NAAD by LND cells and controls ($n = 5$) was highly significant ($P < 0.005$), but not into NAD ($P = 0.7$); (**b**) [^{14}C]uridine into UTP, or [^{14}C]cytidine into CTP, by LND cells compared with controls ($n = 8$) was not significantly different ($P = 0.18$ and 0.46, respectively), but incorporation of cytidine into UTP by LND cells was significantly lower than controls ($P < 0.005$), and (**c**) [^{14}C]uridine or [^{14}C]cytidine into UDPS was significantly lower in LND cells versus controls with both substrates ($P < 0.005$ and 0.05, respectively).

NAD glycohydrolase (Figure 2). By contrast, cytidine actually increased NAD slightly in LND cells (11%), with little effect on the controls. Since both uridine and cytidine salvage are ATP-dependent, the results suggest a role for UTP in regulating NAD synthesis in fibroblasts, which requires further study. Nicotinic acid did increase NAD slightly in both LND and control cells (17 and 13%), without affecting other nucleotides.

Extent of radiolabel incorporation by LND versus control cells

The two novel findings in the [^{14}C]nicotinic acid studies were that: (i) incorporation by LND cells was 60% greater than for the controls (Figure 3), with no unmetabolized substrate remaining, compared with up to 20% in the controls, and that (ii)

incorporation into the NAD intermediate, nicotinic acid adenine dinucleotide (NAAD, Figure 2), by LND cells was nearly 6-fold greater than controls where incorporation into NAAD was very low, this difference being very significant ($P < 0.01$; Figure 4a).

The reverse was the case for the pyrimidine nucleosides, both being salvaged more actively by controls (LND, 60 % of the control), with [^{14}C]uridine incorporation being mainly into UTP and UDPS. Only 4 and 2 %, respectively, was incorporated into CTP, confirming little activity of CTP synthetase in either control or LND fibroblasts (Figure 3). Although the difference between incorporation by LND compared with controls did not reach significance ($P = 0.18$), incorporation into UDPS by LND cells was significantly lower ($P < 0.005$; Figures 4b and 4c).

LND cells incorporated slightly more [^{14}C]cytidine (82 %) than uridine compared with controls, with 50 % being incorporated into CTP. The remainder was deaminated to uridine (Figure 3), although total incorporation overall was lower than that of uridine. In controls, proportionately less radioactivity was found in CTP, with more being deaminated and incorporated into UTP and UDPS (Figure 3). The difference between the mean incorporation into CTP by LND compared with control fibroblasts was not significant ($P = 0.45$), but again, as for uridine, cytidine incorporation into UDPS by LND cells was significantly lower ($P < 0.005$; Figure 4b and 4c).

Given that the incubation conditions were the same for all three precursors (in terms of radiolabel specific activity and cell number), very different total amounts of radioactivity were incorporated in the above studies, even by control fibroblasts (Figure 3). [^{14}C]Uridine incorporation was twice that of cytidine and four times that of nicotinic acid, confirming the importance of uridine salvage for human fibroblasts. Compared with controls the LND cells always showed a higher uptake of nicotinic acid, but lower uptake of pyrimidines (Figure 3), with twice as much unmetabolized uridine or cytidine being found in LND cells (12 and 14 %, respectively), compared with the controls (6 and 9 %; results not shown). By contrast, free nicotinic acid was found only in controls, suggesting that high PPRibP concentrations are crucial for uptake and incorporation of nicotinic acid into pyridine nucleotides by LND cells.

DISCUSSION

The novel finding here is the severe NAD depletion in LND fibroblasts, mean concentrations being 50 % of controls cultured under the same conditions, together with a similar depletion of ATP and GTP. The latter has been reported in one other study in lymphoblasts [6]. The enhanced [^{14}C]nicotinic acid incorporation by LND fibroblasts compared with controls would support deranged pyridine nucleotide metabolism in LND. The 6-fold greater incorporation of [^{14}C]nicotinic acid into NAAD by HPRT$^-$ cells confirms enhanced activity of nicotinic acid phosphoribosyltransferase, as noted in LND erythrocytes, presumably due also to the elevated PPRibP [28,29]. Consequently, elevated, not low, NAD concentrations might have been expected. However, since little ADP ribose was detected (Figure 3), NAD depletion in LND fibroblasts here must relate to further utilization for poly(ADP-ribose) synthesis (Figure 2) [30,31]. The latter could explain the apparent contradiction between the low fibroblast NAD in LND fibroblasts compared with the elevated concentrations in erythrocytes in LND patients relative to controls. The anucleate erythrocyte lacks poly(ADP-ribose) polymerase activity [31].

The concomitant reduction in ATP and GTP, but normal pyrimidine nucleotides, also differs from most reports, with the exception of the one study in HPRT$^-$ lymphoblasts [6,18].

However, no significant difference in NAD concentrations was reported in the latter, presumably reflecting the more actively dividing cell type used [6]. We believe the explanation for the low ATP and GTP also found here in LND cells, as proposed by McCreanor and Harkness [6], must be culture conditions, which in fibroblasts affected pyridine nucleotides as well. The importance of culture conditions in determining nucleotide concentrations was detailed in the Introduction and stressed also in studies highlighting a new developmental disorder with increased cellular nucleotidase activity [23]. Differences compared with controls were apparent only when fibroblasts were forced into maximum purine production: purine-free medium, dialysed serum, logarithmic-phase growth and frequent medium changes. Certainly, in Friend cells, medium changes at different stages of growth or differentiation have profound and diverse effects on poly(ADP-ribose) synthesis [32]. Importantly, slow-growing fibroblasts, as in this study, are known to have lower nucleotide concentrations than rapidly growing fibroblasts. Thus not only cell type but also growth rate may have played a role in highlighting the differences observed here.

The equally interesting findings relating to pyrimidine metabolism are, first, that although pyrimidine nucleotide concentrations (UTP, CTP) in LND fibroblasts were similar to controls, pyrimidine salvage was more active in controls than in LND fibroblasts, differences which would not have been apparent using undialysed non-heat-inactivated FBS. Second, in both LND and control fibroblasts, CTP synthesis was principally by salvage, little [^{14}C]uridine (< 4 %) being converted into CTP by CTP synthetase. This contrasts with the high CTP synthetase activity in lymphoblasts [33], which must affect both uridine and cytidine nucleotide concentrations. Third, cytidine was salvaged into CTP with similar efficiency by both cell types, although less was degraded to uridine nucleotides by LND cells. Nevertheless, deamination (greater than 50 % in both) was the principal route of metabolism. Thus although human fibroblasts do lack CTP synthetase they clearly contain an active cytidine deaminase. In this respect they differ likewise from lymphoblasts and mitogen-stimulated lymphocytes [24,27]. Similar differences may underlie the absence of evident NAD depletion in HPRT$^-$ lymphoblasts [6].

The essential question here is what is the activity of all these enzymes in human brain, especially in the neonatal period [34]? If the fibroblast model is more relevant, then the significantly lower salvage of either uridine or cytidine into UDPS by LND fibroblasts could be extremely important in the pathogenesis of LND. Normal brain function and metabolism have an absolute requirement for uridine and cytidine [35]. Cytidine is vital for neuronal phosphatidylcholine synthesis, and uridine is needed for galactosides [36,37]. Moreover, although there are no comparable data for humans, pyrimidine salvage in brain also reportedly increases in parallel with growth in neonatal rats, *de novo* synthesis being minimal thereafter [36,38].

Assuming that fibroblasts are a more accurate model for the situation in human brain, the combined results question whether, or how, a depletion of NAD coupled with (or in addition to) the ATP and GTP depletion also noted here could be implicated in the neurological deficits in LND. McCreanor and Harkness [6], focusing on the early death in LND from respiratory failure, from the correlation between ATP depletion and reduced poly-(ADP-ribose) synthetase activity in HPRT$^-$ lymphoblasts, implicated pyridine nucleotide metabolism indirectly in the respiratory symptoms. The consequence would be reduced DNA repair [6]. This proposal is based on the fact that with little replication in human brain cells after the first year of life, DNA repair accounts for most of the DNA turnover; repair defects involving pyridine

nucleotide metabolism are known to be associated with neurological deterioration [39].

A parallel may be drawn with the studies of ours and others in adenosine deaminase (ADA) deficiency [40,41]. In ADA deficiency, a combination of events involving NAD and ATP depletion results in reduced poly(ADP-ribose) synthesis and restriction of DNA-strand break repair, with eventual apoptosis producing severe lymphopenia [40]. Long-term follow-up studies in bone-marrow-transplanted ADA-deficient patients have demonstrated cognitive and behavioural abnormalities not present in non-ADA transplanted immunodeficient children [41]. These neurological problems were attributed to pre-transplantation neuronal cell death via apoptotic mechanisms similar to those in lymphocytes; an hypothesis supported by *in vitro* and *in vivo* studies in chick embryonal neurons [42]. Thus similar small and possibly short-lived reductions in vital nucleotides such as NAD and ATP *in vivo*, as demonstrated here, if reflected in the central nervous system, may be relevant to the pathogenesis in LND under the specific conditions pertaining very early in brain development. The rate of brain growth in the human neonate is reportedly rapid [43], HPRT activity seemingly increasing similarly at this stage, coinciding with the period of greatest activity in protein, DNA and synapse formation: any growth failure at this point could not be corrected later [43]. In support of this hypothesis a recent neuro-imaging study confirmed that LND patients are indeed microcephalic and maturational arrest was proposed, associated with a compensatory increase in skull thickness [12]. Of relevance to this argument may be the observations that p53 expression is required for the thymocyte apoptosis occurring in ADA deficiency, whereas p53 itself is a cellular regulator of GTP synthesis at the level of inosine monophosphate dehydrogenase, the only remaining route of GTP synthesis available in LND cells [44,45].

GTP depletion, likewise noted here, may, as proposed by Watts [10], be implicated in the neurobehavioural abnormalities in LND by compromising the production of, and balance between, the neurotransmitters dopamine and 5-hydroxytryptamine (serotonin) [9,10]. Certainly, the post-natal reduction in brain purine *de novo* synthesis would leave salvage as the principal route of GTP synthesis even for normal neuronal cells [46]. The importance of salvage for sustaining GTP concentrations is evident from the GTP depletion in erythrocytes of LND patients where ATP is normal [25]. If the erythrocyte reflects the situation in LND brain, then GTP depletion could be involved in one of three ways. First, restriction of the GTP supply essential for GTP-cyclohydrolase activity could mimic the symptoms in hereditary progressive dystonia [10,47,48]. In this disorder abnormalities in the GTP-cyclohydrolase gene disrupt both the dopamine receptor and 5-hydroxytryptamine pathway [10]. Support for this possibility comes first from the sensitive neuro-imaging techniques mentioned above, which also noted reduced dopamine transporter density linked to the movement disorder in LND [11,12]. Second, the earlier results of Lloyd et al. [9] proposed that the neurological manifestations in LND are related to decreased activity of the dopaminergic circuits in the basal ganglia. Third, recent *in vitro* studies in primary cultures from brains of HPRT-knockout mice recorded decelerated rates of dendrite outgrowth from dopaminergic neurons compared with controls [49].

In summary, these studies in LND fibroblasts demonstrate severe depletion of NAD, in addition to the ATP and GTP depletion noted in one previous report in lymphoblasts [6]. The results suggest that (i) culture conditions may be important in any such *in vitro* model, especially one designed to mimic the situation in neuronal cells at an early developmental stage in

human brain and (ii) the cell type used appears to be extremely important in defining differences in salvage of both pyridines and pyrimidines in LND compared with controls. Blasting lymphocytes, unlike brain, have grossly up-regulated purine and pyrimidine synthetic routes. They can also, unlike fibroblasts, convert uridine nucleotides into cytidine nucleotides [33]. Given the demonstrated involvement of GTP depletion in the neurological deficits in hereditary dystonia [10], and the role implied for NAD/ATP depletion in the behavioural abnormalities in transplanted ADA-deficient patients [41], the possibility that depletion of all three nucleotides could underlie at least some of the pathophysiological mechanisms in LND requires further study.

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